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STUDIES ON THE DIETARY REQUIREMENTS OF GUINEA PIGS[1]

I. EFFECTS OF NATURAL VERSUS SYNTHETIC SOURCES OF VITAMIN C

II. EFFECTS OF ROUGHAGE

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Theoretically, a diet suitable for use in a vitamin C bioassay must, when supplemented with vitamin C, be nutritionally complete. Thus far this aim has not been attained in this laboratory. The basal feed mixture which we have developed for assay purposes, though apparently permitting good growth of young animals when supplemented with vitamins A, D, E and C, is not fully equal to the basal feed plus greenfeed. This has been indicated in previous studies by somewhat slower growth but particularly by a successful reproductive record of about 67% as against 80% when greenfeed was used.

One of the features of the Macdonald V diet was the absence of unground roughage, and since guinea pigs possess functional caeca, and in our experience have shown a craving for such material as wood or hay stems, it was considered worthwhile to investigate this factor. It seemed possible that the absence of unground roughage might interfere with or modify normal caecal function as it is known to affect rumen function in ruminant herbivora.

In addition to the need for roughage, the question of the amount and source of vitamin C may be raised. It has been shown by Davey (1921), Dann (1935), Zilva (1936), Bourne (1942), Sullivan (1944) and Kuether (1944), and further confirmed here, that approximately 2 mg. of ascorbic acid (or its equivalent in juices) will prevent the gross symptoms of scurvy in assay-size guinea pigs. It is known that inadequate levels of vitamin C will interfere with reproduction (citations, Warkany, 1945). According to Kramer (1933), and also on the basis of their weights as compared to that of assay pigs, it seemed likely that 5 mg. per day of vitamin C should be adequate for breeding females.

This test was designed, therefore, with a view to ascertaining the need for roughage in the diet, and to observing the relative efficacy of vitamin C from several sources. In general, reproductive response was used as the major criterion of nutritional adequacy.

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METHODS OF INVESTIGATION

Design of the Test

Table 1 presents the general allotment plan employed. The trial was actually conducted in 2 replicates, using 5 females per group in each.

TABLE 1.—ALLOTMENT PLAN FOR THE STUDY OF SOURCES OF VITAMIN C AND ROUGHAGE EFFECTS

Roughages	Sources of Vitamin C			
	Ascorbic acid	Synthetic orange juice	Fresh orange juice	Fresh lemon juice
Nil	10 females*	10 females	10 females	10 females
Dry roughage	10 females	10 females	10 females	10 females
Greenfeed	10 females	10 females	10 females	10 females

*Five pregnant females on each of 2 replicates.

TABLE 1 (a).—EFFECTS OF REPLICATES, ROUGHAGES AND SOURCES OF VITAMIN C ON REPRODUCTIVE RESPONSE

Effects	No. females on test	No. successful pregnancies	No. haemorrhages	No. abortions	No. stillborn	No. litters containing stillborn	No. young/litter (aver.)	Birth weight (aver.)	2-Week gains (aver.)
								(gm.)	(gm.)
Replicate 1	60	42	6	8	24	16	2.8	103	96
Replicate 2	60	55	2	0	24	15	3.4	97	73
Nil	40	27	5	5	7	6	3.0	97	64
Coarse hay	40	33	3	3	21	13	2.9	99	83
Fresh grass	40	37	0	0	20	12	3.4	102	92
Ascorbic acid	30	23	3	3	19	12	3.2	100	87
Artificial orange juice	30	23	0	4	10	5	3.3	100	79
Orange juice	30	27	1	0	10	7	3.1	96	79
Lemon juice	30	24	4	1	9	7	2.9	102	85

Animals and Management

The females used in the first test had previously had either 2 or 3 litters in the breeding colony. They had been wintered on coarse, dry roughage and aqueous ascorbic acid along with the Macdonald V basal diet, and then had received fresh lawn clippings for 4 to 5 weeks prior to going on test. However, mature females were not available for the second replicate so young virgin females, averaging about 608 gm. initial weight were used. These received fresh grass from weaning until going on test.

The groups of animals were confined in a series of open-top cages 12 in. deep by 22 × 33 in., mounted on legs to raise them about 30 in. off the floor of the laboratory. Each cage was supplied with a sliding grid floor made of flat metal in which holes $\frac{5}{8}$ in. square were punched at intervals, leaving a grid of $\frac{1}{4}$ in. between perforations. On this floor were placed the feed and water containers and a galvanized, solid-bottom, iron tray (22 × 16 × 2 in.) containing a layer of wood shavings.

Diets

The basal diet fed, known as the Macdonald V diet, has the following percentage composition:

Oats	15.0
Wheat	13.0
Beet pulp	25.0
Oilmeal	12.5
Skimmilk	15.0
Fishmeal	5.0
Brewers' dried yeast	10.0
Bone char	4.0
Salt (0.1% KI)	0.5

This mixture was ground, mixed, and pressed into small pellets about $\frac{1}{8} \times \frac{3}{16}$ in. in size, and together with water, was fed *ad libitum* to all lots.

During the first test, the fat soluble vitamins A, D and E were fed weekly in 0.4 cc. of corn oil so as to provide 425 i.u. of vitamin A (from concentrated ling oil), 48 i.u. of vitamin D_2 (calciferol) and 3 mg. of alpha-tocopherol per day. For reasons to be discussed later, these vitamins were doubled in the second replicate.

TABLE 1 (b).—EFFECTS OF ROUGHAGES AND SOURCES OF VITAMIN C ACCORDING TO REPLICATES

Effects	No. females on test	No. successful pregnancies	No. of haemorrhages	No. abortions	No. of stillborn	No. litters containing stillborn	No. of young/litter (aver.)	Birth weight (aver.)	2-Week gains (aver.)
								(gm.)	(gm.)
Nil									
Replicate 1	20	9	5	5	2	2	2.8	96	79
Replicate 2	20	18	0	0	5	4	3.2	96	57
Hay									
Replicate 1	20	15	1	3	10	8	2.6	96	89
Replicate 2	20	18	2	0	11	5	3.2	101	79
Grass									
Replicate 1	20	18	0	0	12	6	3.1	110	108
Replicate 2	20	19	0	0	8	6	3.7	95	80
Ascorbic acid									
Replicate 1	15	11	1	3	9	7	3.3	98	100
Replicate 2	15	12	2	0	10	5	3.1	101	75
Artificial orange									
Replicate 1	15	9	0	4	8	4	3.3	105	85
Replicate 2	15	14	0	0	2	1	3.4	97	57
Orange									
Replicate 1	15	12	1	0	3	1	2.4	103	96
Replicate 2	15	15	0	0	7	6	3.7	92	68
Lemon									
Replicate 1	15	10	4	1	4	4	2.4	105	96
Replicate 2	15	14	0	0	5	3	3.3	100	80

TABLE 1 (c).—EFFECTS OF SOURCES OF VITAMIN C ACCORDING TO ROUGHAGE TREATMENTS

Effects	No. females on test	No. successful pregnancies	No. of haemorrhages	No. abortions	No. of stillborn	No. litters containing stillborn	No. of young/litter (aver.)	Birth weight (aver.)	2-Week gains (aver.)
								(gm.)	(gm.)
Ascorbic acid									
Nil	10	7	1	2	1	1	3.6	106	68
Hay	10	7	2	1	7	7	3.2	96	95
Grass	10	9	0	0	11	4	3.7	98	94
Artificial orange									
Nil	10	7	0	2	2	2	3.3	93	64
Hay	10	7	0	2	5	1	3.0	102	84
Grass	10	9	0	0	3	2	3.7	104	85
Orange									
Nil	10	8	1	0	3	2	3.4	89	56
Hay	10	10	0	0	6	1	2.8	100	77
Grass	10	9	0	0	1	4	3.2	98	98
Lemon									
Nil	10	5	3	1	1	1	2.6	98	68
Hay	10	9	1	0	2	4	2.9	98	83
Grass	10	10	0	0	6	2	3.1	106	94

Vitamin C Supplements

Ascorbic acid, dissolved in distilled water immediately prior to dosing, was given daily, with the aid of a calibrated syringe, at the rate of 5 mg. per day to those animals receiving this supplement.

A 'synthetic' orange juice, resembling natural orange juice in major characteristics, was prepared from the following ingredients (Radford *et al.*, 1937):

Buffer mixture	74 gm.
Sugar (sucrose)	396 gm.
Orange extract	5 cc.
Water to make	128 oz.

The buffer mixture consisted of:

Calcium phosphate: $CaHPO_4 . 2H_2O$	1.075%
Calcium citrate: $Ca_3(C_6H_5O_7)_2 . 4H_2O$	4.57
Sodium phosphate: Na_2HPO_4	3.83
Citric acid: $C_6H_3O_7 . H_2O$	59.08
Sodium citrate: $Na_3C_6H_5O_7 . 2H_2O$	12.75%
Corn starch[1]	18.62
Sunset yellow	0.05

The orange extract was a solution of 4.57% orange oil, plus 95.43% of 95% ethyl alcohol.

At the time of dosing, crystalline ascorbic acid was added to a portion of this juice so as to supply each animal 5 mg. per day in a 10 cc. dose.

[1] Later replaced by an equal quantity of dextrin so as to remain in solution.

The natural juices, orange and lemon, were extracted by pressure and fed to give 5 mg. per day on an assumed average potency of 50 mg. per 100 cc. of juice—an hypothesis which appeared justifiable from available tables of citrus fruit composition (Stevens *et al.*, 1945). Chemical assays were run periodically to establish the actual potency.

Because of the difficulty experienced in replicate 1 in administering such large doses of lemon juice it was decided to remove the citric acid for the next test. The method of decitrating cited by Harden and Zilva (1918) was modified and adopted for this work.

The average values for vitamin C content, obtained by chemical assay during the test are shown in Table 2.

TABLE 2.—SUMMARY OF CHEMICAL ASSAYS FOR VITAMIN C

(mg./100 cc.)

Kind of juice	Total ascorbic acid	Dehydroascorbic acid
Fresh orange	50–57[1]	
Fresh lemon (Replicate 1)	40–54	
Fresh lemon (Replicate 2)	30–45	0– 6
Fresh decitrated lemon	25–43	0– 8
Stored decitrated lemon	25–35	8–15

[1] Assay somewhat lower in the second replicate.

Roughages

The greenfeed, when provided, consisted of fresh lawn clippings except for the latter part of the second replicate when grass became unavailable and raw cabbage was substituted. About 3 oz. of fresh grass or cabbage per animal was allowed.

Records

During the course of the experiment the following data were recorded:

(*a*) Gains of the breeding females, and feed consumption by weekly weighings.

(*b*) Reproductive failures as measured by uterine haemorrhages, abortions, and stillborn young.

(*c*) The number of young per litter and the individual birth weights.

(*d*) Weight gains of the young during the nursing period.

(*e*) Post-mortem findings on all females which died or were killed because of poor health.

Analysis of the Data

Where the number of observations on any criterion was considered sufficient, an analysis of variance was conducted according to the methods of Pearson and Bennett (1942). The variance scheme follows:

PARTITION OF VARIANCE AND DEGREES OF FREEDOM

Source	Dam's weights, litter weights, no. young/litter	Birth weights and gains of young
Total variation	95	257
Between sub-groups	23	23
Replicates	1	1
Roughage	2	2
Sources of vitamin C	3	3
Replicates × roughages	2	2
Replicates × sources	3	3
Roughage × sources	6	6
Second order interaction	6	6
Error	72	234

A complete summary table of the average results appears in Table 1 ((a), (b), (c)). For clarity the detailed discussion of findings will be presented under several sub-headings.

Method of Removing Citric Acid from Lemon Juice

The citric acid was removed from solution by adding an excess of $CaCO_3$ with constant stirring, after which two volumes of absolute ethyl alcohol were added to precipitate the calcium citrate as a gelatinous mass. The mixture was filtered through buchners and the precipitate was washed with absolute alcohol and filtered again. The combined filtrates were then evaporated in vacuo at 35–40° C., with CO_2 gas constantly bubbling through. By calculating the loss of alcohol and juice in the residue from the dilutions, and quantities of wash alcohol recorded, it was possible to reconstitute the volume of lemon juice with a reasonable degree of accuracy.

Sufficient juice for a week's supply was decitrated once weekly and stored under a layer of corn oil in a refrigerator box.

RESULTS AND DISCUSSION

Differences Observed between Replicates

As seen in Table 3 several important differences became evident as a result of unavoidably varied conditions between replicates. There was on the whole, a better reproductive response in Trial 2 but somewhat slower gains of the young during nursing. It is doubtful if the increase in fat-soluble vitamins was responsible for the higher reproductive success in the second test because the aborted foetuses, still-born young, and dams, post-partum, did not display symptoms considered characteristic of deficiencies of these vitamins. More likely, many of the females of Trial 1, which had recently borne litters were subjected to dietary conditions on the poorer diets which their bodily stores could not remedy in order to maintain pregnancy.

There were differences between birth weights, number of young per litter, and gains of young during the nursing period, which by statistical

analysis proved real with odds at 19 : 1. It is well to mention in this connection that the correlation between birth and weaning weights has proven highly significant in previous work in this laboratory. Collier (1942) found the correlation coefficient (r) to be 0.485 to 0.640 for 127 degrees of freedom. The litter sizes may further reflect a condition of depletion in the females of the first test.

TABLE 3.—RESPONSES OBTAINED FROM MATURE FEMALES (REPLICATE 1) AND FROM VIRGIN FEMALES (REPLICATE 2) IRRESPECTIVE OF DIET

Index	Replicate No. 1	Replicate No. 2
Initial weights	841 gm.	608 gm.
% Successful litters	70%	92%
No. haemorrhages	6	2
No. abortions	8	0
% Stillborn young	16%	11%
Birth weights	103 gm.	97 gm.
No. young/litter	2.8	3.4
2-Week gains of young	96 gm.	73 gm.
Basal feed consumption[1]	40 gm.	39 gm.

[1] Pellet consumption irrespective of whether or not roughage was allowed.

Effects of Sources of Vitamin C

With respect to the effects of sources of vitamin C on disturbances of reproduction it was noted (Table 4) that aqueous ascorbic acid permitted more reproductive failures (abortions and haemorrhages) than any of the other 'synthetic' or natural juices. It may be noted, however, (Table 1 (a)), that none of these failures occurred in the second replicate *except* with ascorbic acid.

TABLE 4.—EFFECTS OF SOURCES OF VITAMIN C ON REPRODUCTIVE BEHAVIOUR

	Ascorbic acid		Synthetic orange juice		Orange juice		Lemon juice	
	1[1]	2	1	2	1	2	1	2
No. of females	15	15	15	15	15	15	15	15
No. of haemorrhages	1	2	0	0	1	0	4	0
No. of abortions	3	0	4	0	0	0	1	0
No. of successful pregnancies	11	11	9	14	12	15	10	14
% Successful pregnancies	77		77		90		80	
% Of litters having stillborn young	40		17		23		23	
% Of stillborn young	20		11		11		11	

[1] First and second replicates indicated.

The natural juices tended to promote a higher proportion of successful litters and living young. The single haemorrhage appearing under orange juice occurred in the first replicate.

The fact that the levels of vitamin C, on the lemon juice, proved appreciably lower than 50 mg. per 100 cc., justifies the conclusion that 5 mg. per day of this vitamin was above the minimum level for pregnant

guinea pigs. Hence the poor response on ascorbic acid-treated lots suggests a deficiency of some factor, other than vitamin C, which has adversely affected the carrying of the foetus to term, but (Table 5) which has not affected the birth weights or post-natal response of the living young.

TABLE 5.—EFFECTS OF SOURCES OF VITAMIN C ON GROWTH, BIRTH WEIGHTS, LITTER SIZES AND FEED CONSUMPTION*

Index	Ascorbic acid	Synthetic orange juice	Orange juice	Lemon juice
	(gm.)	(gm.)	(gm.)	(gm.)
Birth weight	100	100	96	102
No. young/litter	3.2	3.3	3.1	2.9
2-Week gains	87	79	79	85
Feed consumption	38	40	39	39

* No comparisons proved significantly different at P = .05.

It becomes obvious that vitamin C, per se, is not the limiting factor in these results. While it is probably true that a deficiency of this vitamin in early pregnancy may lead to abortion (Moriquand, 1935; Kramer, 1933; and Warkany, 1945) pregnancy is a criterion complicated by several nutritional and other factors. Consequently the efficacy of vitamin C in various mixtures could be compared through pregnancy tests only on a level of the vitamin considered to be at, or slightly below, the minimum level. In this way a gradient of response would become established using reproductive failures, along with gross and post-mortem scurvy diagnosis. Therefore, since Kramer was unable to obtain living litters when feeding 5 cc. of orange juice daily, it appears that with the Macdonald V diet the level necessary for pregnancy lies between 2 and 5 mg. per day.

Effects of Roughages

The salient points relating to these factors are depicted in Table 6. Evidently fresh greenfeed completely fortified the diet since no abortions or haemorrhages occurred when it was fed. That roughage in either form was beneficial is also evident.

Since the females were not conditioned on their respective diets for more than an average of 10 days prior to conception, no difference in litter size could be expected unless resorption of some of the foetuses in the litters occurred. The trend shown in birth weights was insignificant but the gains made by the young favoured the use of roughage. This may be due to greater feed consumption by the young themselves.

The decreased amount of feed (pellets) consumed when roughage was available was more than balanced by the weight of roughage consumed. The actual energy intake, however, was probably about maintained if the dry roughage was 45% digestible (Crampton *et al.*, 1940), and if the dry matter of the greenfeed (at 75% moisture) was somewhat more digestible, as was doubtless the case.

TABLE 6.—EFFECTS OF TYPES OF ROUGHAGE FED ON REPRODUCTIVE BEHAVIOUR

Index	Nil	Dry roughage	Greenfeed
Reproductive failures	10	6	0
% Successful litters	68	83	92
No. young per litter	3.0	2.9	3.4
Birth weight (gm.)	97	99	102
2-Week gains (gm.)	64	83	92
Dam's weight loss in nursing period (gm.)	46	21	34
Daily feed consumption (gm.)	44	37	37
Estimated roughage consumed/pig/day (gm.)[1]	0	25	80[2]

[1] Based on measurements taken at intervals during the test.
[2] Moist weight as fed.

SUMMARY AND CONCLUSIONS

Fresh orange juice, lemon juice (decitrated) and synthetic orange juice protected the pregnant guinea pigs from haemorrhages and abortions to a greater extent than did aqueous ascorbic acid.

A trend exists to the effect that a greater number of females gave birth to one or more living young when natural juices were fed.

The minimum level of vitamin C for pregnancy in guinea pigs does not exceed 5 mg. per day.

Fresh greenfeed completely supplemented the Macdonald V basal diet for pregnant guinea pigs, and well-cured, stemmy hay appeared to constitute a beneficial addition to the basal diet.

The differences observed between the responses of females in their first pregnancy and those in third or fourth pregnancy, were apparently attributable to the partial depletion of essential nutrients resulting from previous gestations.

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RAPID FIELD TESTS FOR THE DIAGNOSIS OF AMERICAN FOULBROOD OF BEES[1]

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Simple, rapid procedures for detecting infection and disease are continually being sought as they save material, space, time, personnel and most important of all, permit early treatment of the disease and thus prevent its dissemination. Field methods are particularly useful in this connection and when supplemented by careful observation of symptoms may constitute an adequate diagnosis for recommending treatment. According to White (3) American foulbrood is the easiest of the brood diseases to diagnose, even from symptoms alone. However, in view of the drastic treatment often recommended for infected comb it is obvious that there should be no question of the correctness of the diagnosis. In this regard the simple field test recently described by Holst (1) should be of great value for inspectors, operators or beekeepers. It is based upon the fact that *Bacillus larvae*, etiological agent of this disease, produces 2 enzymes one of which curdles milk whereas the other digests the curd. Holst emphasized liquefaction of the curd as being the characteristic feature of American foulbrood which distinguished it from all other larval diseases and from abnormal conditions of bees. Simple as the test undoubtedly is, it still involves use of homeopathic vials, skimmilk powder, reconstitution of the milk, and measuring aliquots of milk and water. In this laboratory, emphasis has been placed on curdling of the milk rather than digestion of the curd, the test requiring nothing more than a piece of glass and 2 drops of whole milk, pasteurized or not. Results of comparative studies with this method, the milk digestion procedure of Holst, microscopic examination, cultural and nitrite tests are presented herein.

MILK COAGULATION TESTS

At first, small glass vials were used, to which were added 1 scale and 8 drops whole milk (raw or pasteurized). The scale was macerated thoroughly for 10–15 seconds and the time required for coagulation noted from the moment the milk was added. Since this procedure rendered the sample unavailable for the cultural work contemplated, another procedure was developed in which scale or ropy material was macerated in 2 drops of lukewarm milk on a glass slide, a firm clot resulting in a shorter time than even with the tube method. The following procedure was then adopted which permitted various tests being made on 1 sample. Single drops of sterile water were placed aseptically in sterile Petri plates (4 drops per plate) and scale or ropy material transferred to it and macerated thoroughly. Several loopfuls of this mixture were transferred to a tube of yeast-carrot-semi-solid agar medium and incubated at 37° C. for morphological examination and nitrite tests; 1 loopful was transferred to a slide for microscopic examination for spores and the remnant exposed to the air to dry. Then,

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TABLE 1.—COAGULATION OF MILK BY A.F.B. AND E.F.B. SCALE AND HEALTHY LARVAE

Nature of material		Microscopic examination	Milk coagulation test	Yeast-carrot-semi-solid agar culture	Nitrite test
			Time		
A.F.B. scale	1	*B. larvae* spores	30 sec.	Typical *B larvae*	+
	2	*B. larvae* spores	15 sec.	Typical *B. larvae*	+
	3	*B. larvae* spores	45 sec.	Typical *B. larvae*	+
	4	*B. larvae* spores	25 sec.	Typical *B. larvae*	+
	5	*B. larvae* spores	30 sec.	Typical *B. larvae*	+
A.F.B. scale treated with formaldehyde gas	6	*B. larvae* spores	No coagulation	No growth	−
	7	*B. larvae* spores	No coagulation	No growth	−
	8	*B. larvae* spores	No coagulation	No growth	−
	9	*B. larvae* spores	No coagulation	No growth	−
	10	*B. larvae* spores	No coagulation	No growth	−
E.F.B. scale	11	*B. alvei* spores	1 min 47 sec.	Typical *B. alvei*	−
	12	*B. alvei* spores	2 min. 25 sec.	Typical *B. alvei*	−
	13	*B. alvei* spores	2 min. 42 sec.	Typical *B. alvei*	−
	14	*B. alvei* spores	3 min.	Typical *B. alvei*	−
	15	*B. alvei* spores	4 min.	Typical *B. alvei*	−
Health larvae	16	No spores	No coagulation	No growth	−
	17	No spores	No coagulation	No growth	−
	18	No spores	No coagulation	No growth	−
	19	No spores	13 min.	No growth	−
	20	No spores	45 min.	No growth	−

2 drops of milk were added, the mixture macerated 10–15 seconds and the time required for a firm clot to develop noted (from the moment the milk was added). Mechanical mixing of the milk and material should be limited to 10–15 seconds, otherwise the curd formed will break up and a firm clot will not be obtained, resulting in a false negative. In the field, mixing of the milk and suspected sample may be accomplished with a match stick, or knife point and like objects. Should less than an entire scale be available, 1 drop of milk will suffice. The glass and adherent material, as well as any metallic object used for macerating, should be boiled for 20 minutes and cleaned; if matches are used they should be collected and burned, thus dissemination of the spores will be reduced to a minimum.

Table 1 presents typical data taken from various experiments comparing the tests mentioned above on A.F.B. scale before and after treatment with formaldehyde gas, European foulbrood scale and healthy larvae. It is obvious that although coagulation of milk is obtained with E.F.B. as well as A.F.B. scale, the time factor is sufficient to separate the two. Microscopic examination of the scale, cultural tests for viable *B. larvae* and nitrite tests confirmed the conclusions suggested by the curdling of the milk. Treatment with disinfectants such as formaldehyde apparently rendered the enzyme system inactive and the test useless; cultural tests are then required to detect viable spores.

Coagulation of the milk by E.F.B. scale was a gradual process as compared with the sudden curdling produced by A.F.B. material. Since curdling occurred with both, however, it was considered necessary to apply

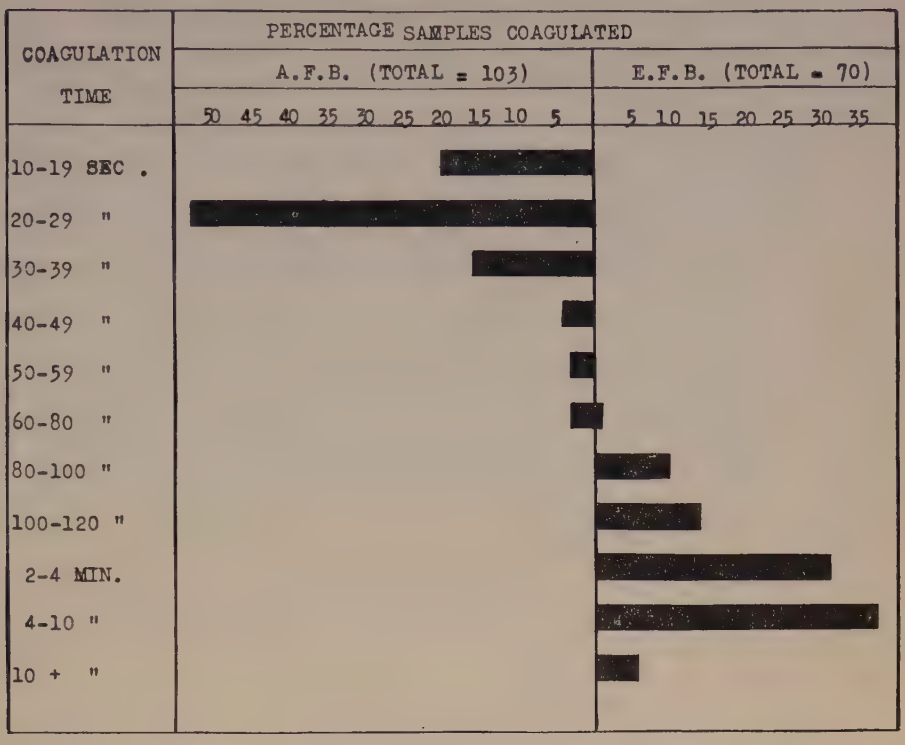

FIGURE 1. Comparative rates of coagulation of milk by American foulbrood and European foulbrood scale.

the test to a larger number of samples. The results given in Figure 1 show that over 90% of the A.F.B. samples curdled milk within 40 seconds, the majority (over 70%) requiring less than 30 seconds, whereas over 90% of the E.F.B. samples required more than 80 seconds, the majority using more than 2 minutes. Overlapping time intervals occurred with about 2 to 3% of the samples tested. These results were not influenced appreciably by the age of the A.F.B. scale (up to one year); however, older E.F.B. scale (over 6 months) required considerably longer periods ranging from 5 to 40 minutes to curdle the milk. It would appear then, that rapid coagulation of milk by suspected scale is a fairly reliable criterion of infection with A.F.B. and one which any beekeeper or inspector can readily use.

MILK DIGESTION TEST

The milk digestion procedure of Holst is more specific though somewhat more involved and is affected by age of material and temperature of the test solution. The test was applied to fresh and old A.F.B. and E.F.B. scale essentially as directed by Holst. To scale in ordinary test tubes were added 20 drops distilled water and 4 drops of a 10% suspension of skimmilk powder. After slight shaking, half of the samples were placed in a water bath at 38° C. and half left at room temperature. The E.F.B. scale did not digest the milk within a 3-hour period. Fresh A.F.B. material

including scale and "ropy" samples, at 38° C. caused rapid digestion of the milk as is shown in Table 2; 80% of the tubes showing distinct evidence of clearing within 20 minutes and 100% within 30 minutes. Tubes with old scale required at least twice as long for the same proportion of samples to produce digestion of the milk at 38° C. At room temperature only 36% of the tubes showed clearing after 1.5, 70% after 2.5 and 100% at 3 hours.

It was also considered of importance to determine at which stage of infection these tests would be applicable. Theoretically, since the enzyme systems involved are produced and liberated during spore formation (2), and in the infected larvae, spores can be demonstrated only in the scale stage and later stages of decay ("ropy" and "pre-ropy" material) (3), the tests should function only with such materials. This was found to be the case in various experiments the results of one of which are given in Table 3. Freshly infected comb provided the material; 20 samples of scale, 20 of ropy material, 20 of dead, shrunken elongated brownish larvae and 20 healthy white larvae were removed and 10 of each set tested by the rapid coagulation and by the digestion techniques. Rapid coagulation and digestion was induced by both "scale" and "ropy" samples and *B. larvae* spores were abundant in these. Neither the dead nor the healthy larvae caused digestion of the milk after 3 hours, but several of the dead larvae caused coagulation within 10 minutes and were characterized by the presence of a few *B. larvae* spores. The healthy larvae did not coagulate the milk.

TABLE 2.—DIGESTION OF MILK BY FRESH AND OLD A.F.B. SCALE AT 38° C.

Time intervals (min.)	% Samples showing distinct evidence of milk digestion	
	Fresh scale	Old scale
10	35	0
20	80	17
30	100	34
40	—	70
50	—	82
60	—	92
70	—	97
80	—	100

TABLE 3.—COAGULATION AND DIGESTION OF MILK BY A.F.B. MATERIAL AT DIFFERENT STAGES OF THE DISEASE

Sample	No. tested	Coagulation time (sec.)		Spore stain	% Samples digesting milk within 30 min.
		Range	Average		
Scale	10	20 – 40	28.7	+	100
Ropy material	10	17 – 60	38.4	+	100
Dead larvae	10	—	Over 10 min.	3 with few spores	0
Healthy larvae	10	—	No coagulation	—	0

CONCLUSION

The simplicity of the milk coagulation test and its economy of time and material strongly recommend it as a field test for A.F.B. Granted that the digestion test is more specific, it nevertheless requires certain equipment, a longer time period and at least body temperature for effective application. It is considered, therefore, that rapid coagulation of milk in conjunction with the usual observations of symptoms is a reliable index of American foulbrood and one which can be performed readily and effectively.

SUMMARY

A rapid, simple field test for A.F.B., based on coagulation of milk, consists essentially of macerating suspected material with 2 drops of milk on a piece of glass and noting the time required for a firm curd to develop.

Although E.F.B. scale was also found to coagulate milk, the time required was considerably longer than for A.F.B. scale, which with 90% of the samples required less than 40 seconds, and with 70% less than 30.

Formaldehyde gas destroyed the enzyme system and rendered the test useless.

Digestion of milk by scale was most rapid with fresh material at body temperature.

Rapid digestion and coagulation of milk occurred only with scale or "ropy" material and not with healthy larvae or those in the early stages of the disease.

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THE DUSTING OF CUT POTATO TUBERS AS A PREVENTIVE AGAINST *PYTHIUM* ROT[1]

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Large losses occur through the rotting of cut potato sets in the coastal regions of British Columbia, particularly when the sets are planted on heavy soils and the planting is followed by wet weather. The cause of the rot was found by Jones[4] to be due to *Pythium ultimum* Trov. He demonstrated that the field losses could be reduced by planting small whole tubers or sets that had been allowed to callous by being kept under humid conditions for 48 hours.

Many growers plant whole tubers of the small grade of certified seed potatoes on areas where previously, through the planting of cut sets, they had experienced heavy losses. However, among the growers using cut sets, few take the precaution of allowing them to callous over under humid conditions. Consequently, 'misses' are far too common, and the cause has usually been traced to *Pythium* rot. On the other hand, the practice of dusting the cut sets with either hydrated lime or flowers of sulphur is quite common, partly because many growers believe that these dusts assist in preventing the sets from rotting, and partly because these dusts prevent the sets from sticking together in the potato planter.

Field observations suggested that neither hydrated lime nor sulphur is a satisfactory preventive against the premature rotting of the seed pieces, hence the influence of various dusts against *Pythium* rot was studied.

A group of potato tubers were inoculated with *Pythium ultimum* and incubated at 25° C. until completely soft. They were then macerated in a Waring blender and thoroughly mixed with the soil on a glass-house bench. Seed pieces were prepared by quartering 6 oz. tubers of the variety Netted Gem. After being thoroughly dusted, these 1·5 oz. sets were planted in the inoculated soil. About 3 weeks later, when the normal plants were approximately 3 in. high, the sets were dug and examined, and recorded as sound or infected. Three successive trials were carried out in which 25 sets were dusted with each of the following 8 dusts. The average percentage of sets that became infected in the 3 trials were as follows: Dithane, 78%; Sulphur, 96%; Hydrated lime, 78%; Arasan, 40%; Fermate, 21%; Spergon, 39%; Copper oxide, 100%; Semesan Bel, 68%; No treatment, 84%. The necessary difference for a 5% level of significance was 18.5%.

The results of the trials show that the common practice of dusting cut tubers prior to planting with hydrated lime or sulphur is without value as a preventive of *Pythium* rot. Likewise, no significant protection was effected by Dithane, Semesan Bel, or red copper oxide. It is noteworthy that, although dusting with Semesan Bel failed to protect the seed pieces from *Pythium* rot, this dust was the only one which appeared to stimulate

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[4] Jones, Walter. Soft rot of potatoes caused by *Pythium ultimum* Trov. Sci. Agr. 15 : 402-410. 1935.

the growth of the plants that survived. The copper oxide appeared to aggravate the rot. Without an exception, all the sets treated with copper oxide became infected, and only one plant in each trial appeared above ground, compared with over 50% in the lots treated with the other dusts. Fermate was significantly superior to all the other dusts, but Spergon and Arasan exerted a satisfactory protective influence. The dusting of freshly cut tubers with Fermate prior to early planting on heavy soils is recommended as a preventive of *Pythium* rot, and to prevent the sets from adhering together in the potato planter.

SUMMARY

The dusting of freshly cut potato sets with Fermate prior to planting effectively prevented the premature rotting of the sets by *Pythium ultimum* Trov. Dusting with Spergon and Arasan was also beneficial, but somewhat less effective. Hydrated lime, sulphur, Dithane, and Semesan Bel were without significant protective effect, but Semesan Bel appeared to stimulate the initial growth of the plants that survived. Dusting with red copper oxide aggravated the rot.

A COMPARISON OF CULTURAL METHODS FOR THE MAINTENANCE OF CERTAIN ECONOMIC FUNGI[1]

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INTRODUCTION

The commonly-employed laboratory method of maintaining cultures of fungi isolated from infected plants involves the use of media rich in nutrients, for example, potato dextrose, malt, or cornmeal agars. The practice followed is to transfer to a fresh tube when the first begins to dry out, and so on. Although the organisms grow well under these conditions and remain viable for a long period of time, for some reason they tend to exhibit genetic instability, in consequence of which mutants appear in the tubes. These may show marked morphological differences from the original or "parent" type, and many of them are able to crowd out the latter, a phenomenon that may be termed "cultural degradation." This process of change has been studied by Miller (9, 11) in *Fusarium*, the conclusion drawn being that the usual cultural media are of doubtful value for maintaining pure cultures of these organisms. Lucas (7) has described "deterioration" of cultures of the red rot fungus, *Colletotrichum falcatum*. Raper and Alexander (13) state that variants arising in cultures of *Penicillium notatum* may displace the original strain. This process constitutes a danger here, since the variants were regularly characterized by reduced penicillin production. Similarly, cultural variants obtained by Miller were less virulent toward the host, and those of Lucas showed loss of sporulation.

It is natural that persons working with fungi in culture will want some assurance that this phenomenon of "degradation," or "running out," will not lead to the loss of the types isolated from nature. This paper constitutes an account of an experiment performed to compare several cultural techniques in regard to their value in maintaining certain fungi without danger of loss due to mutation.

MATERIALS AND METHODS

The organisms employed were: *Thielaviopsis basicola* (Berk.) Ferraris, isolated from tobacco plants affected with black root rot; *Septoria glycines* Hemmi, from "brown spot" lesions on soybean leaves; and *Penicillium notatum* Westling.[5] The muskmelon *Fusarium*[6] was included in order to relate the results to preceding studies on this organism by Miller (9, 10, 11).

Three types of culture media were employed: potato dextrose agar, prepared according to the formula of Riker and Riker (15); soil infusion agar, as prepared by Miller (9); and moist, sterilized soil. The agars were

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[5] Culture obtained from the Banting Institute, University of Toronto, through the courtesy of Dr. P. H. Greey. The strain was derived from Fleming's original culture and is known as 1249B21.

[6] No species name is appended here for the reasons given by Miller (11, 12). In brief, no satisfactory systematic treatment is available for the organisms grouped in the section Elegans of the genus *Fusarium*.

dispensed in the form of test-tube slants, and the soil was added to test-tubes to within an inch of the top. These were then moistened, plugged with cotton, and sterilized with steam.

Single-spore and hyphal-tip cultures were made by the biscuit-cutter method of Keitt (5), except that no "spatula" was found necessary. Previous to inoculation of the culture tubes in the experiment, all four of the organisms were purified in this manner, and from young, growing, single-spore cultures suspensions in sterilized, distilled water were prepared. The organisms were introduced into the culture tubes in this form, following which, as a check on purity, at least 40 single-spore or single-hypha isolations were made from all 4 suspensions. With each of the 4 organisms all the cultures thus obtained were identical.

EXPERIMENTAL

Slants of potato dextrose agar and soil infusion agar and tubes of sterilized soil were inoculated in the manner described, and all were transferred a total of 5 times in 9 mo., with the exception of 1 set of soil tubes which was not transferred. There were 4 replicates of each combination of organism and cultural treatment. At the end of this period, the fungus population in the tubes was studied by making single-spore or hyphal-tip isolates from each tube. Suspensions of conidia and mycelial fragments were prepared from the slant cultures by adding sterilized, distilled water and shaking vigorously. The suspensions were then diluted if necessary and streaked on potato dextrose agar plates from which single germinating conidia or hyphal fragments could be removed a day or two later. In the case of the soil tubes, this was accomplished by sprinkling a sample of the contents finely over the surface of potato dextrose agar in Petri dishes. Single hyphae growing out of the grains of soil were transferred to other plates by the biscuit-cutter method. The cultures thus obtained were compared with regard to the characteristics expressed on potato dextrose agar. In certain instances it seemed advisable to continue the experiment, and the tubes concerned were transferred 4 times during the succeeding 6 mo., following which the population in each was again sampled.

The results of this experiment are summarized in Table 1. It will be noted that, in general, fewer mutant cultures were obtained in soil infusion agar than in potato dextrose agar, but fewer appeared in soil tubes than in either of the other media.

Thielaviopsis basicola: This organism failed to remain viable in either the transferred or the non-transferred soil tubes, probably because it was unable to withstand dryness for very long. Although the soil in the tubes was always moist at the time of inoculation, it became quite dry in 2 or 3 wk. Since at no time did more than 8 wk. elapse between transfers, it would seem that they would have to be more frequent if this organism is to be maintained by the soil tube method.

The mutants that appeared in potato dextrose agar and in soil infusion agar differed from the parent type in cultural characters and in the abundance and type of sporulation. There was a tendency for the mutants to be lighter in colour than the parent type. Most of them produced fewer endoconidia, and the chlamydospores, though always present, in some

TABLE 1.—PERCENTAGE INCIDENCE OF MUTATION IN 4 DIFFERENT FUNGI AS RELATED TO FREQUENCE OF TRANSFER AND TYPE OF MEDIUM

Fungus		Medium						
		Potato dextrose agar	Soil infusion agar		Soil tubes			
		5 transfers in 9 mo.	5 transfers in 9 mo.	9 transfers in 16 mo.	5 transfers in 9 mo.	9 transfers in 16 mo.	No. transfers	
							9 mo.	16 mo.
Thielaviopsis	1	13*	100	—	—	—	—	—
	2	100	14	—	—	—	—	—
	3	100	0	—	—	—	—	—
	4	20	—†	—	—	—	—	—
Septoria	1	100	100	100	0	20	—	—
	2	100	100	100	0	92	—	—
	3	100	100	100	0	0	—	—
	4	100	100	100	0	33	—	—
Penicillium	1	93	0	4.5	0	0	0	0
	2	100	0	0	0	28	0	0
	3	100	0	0	0	19	0	0
	4	48	0	9	0	0	0	0
Fusarium	1	100	75	—	0	100	0	0
	2	100	100	—	0	18	0	0
	3	100	86	—	21	100	0	0
	4	100	100	—	0	18	0	0

* Each figure represents an average of 18 isolations.
† Contaminated.

cases were shorter with fewer segments than those produced by the parent type on the same medium. About half of the mutants were characterized by a comparatively slow growth rate on potato dextrose agar. They were also found to differ from the parent type in respect to virulence to tobacco. Inoculum was prepared by growing the parent type and 5 mutant cultures separately in flasks of cornmeal-sand medium for 20 days. This was added to sterilized soil in which tobacco seed (variety Judy's Pride) was then planted. The parent type caused far more serious disease incidence than did any of the mutants. In fact, the latter had no marked effect on the stand of seedlings in the flats, as compared with the non-infested checks. It was found possible, however, to re-isolate two of the mutants from discoloured roots in their respective flats. On the whole, the roots of seedlings growing in soil infested with the mutants showed no macroscopic evidence of infection.

Johnson and Valleau (4) obtained variants from sectors that appeared in isolates of *Thielaviopsis*, and when these were tested for virulence to tobacco, definite symptoms of black root resulted but no marked differences in virulence were noted among the 8 strains employed. They do not state whether an original isolate was included in the experiment. There is no doubt, however, that in their experiment the virulence of the cultural variants was greater than that noted here. Possibly their method of

inoculation—dipping the root systems of seedlings in water suspensions prepared from growing cultures as they were transplanted to sterilized soil—rendered the host more susceptible than the technique employed here.

But these observations are in general agreement with those of Miller (9, 10) who noted that cultural variants of *Fusarium* were consistently lower in virulence than the wild type and they serve to emphasize the importance of maintaining the original isolates in a pure state.

Septoria glycines: This organism remained viable in the transferred soil tubes but could not be recovered from those that had not been transferred. Evidently, then, the organism is unable to survive as long as 9 mo. in dry soil. This point was checked further, and it was found that 6 mo. was approximately the limit of viability under these conditions.

From Table 1 it is evident that the organism was very unstable on both potato dextrose agar and soil infusion agar, but after 9 mo. in sterilized soil the original type was still apparently pure. Six months later, however, variants were isolated from 3 of the 4 replicates.

The mutants showed a wide range of variation, from white, sterile, albino types to dark cultures that were not much different from the parent type. In fact, some of the latter even sporulated more abundantly than the parent type on potato dextrose agar. In general, the mutants obtained from soil infusion agar and from the soil tubes resembled the parent type more closely than did those that appeared on potato dextrose agar, although there were many exceptions in this respect.

Penicillium notatum: It is interesting to note that this organism proved to be the most stable of the four. Even from soil infusion agar after 15 mo. only a very few mutants were obtained, but on potato dextrose agar it seemed about as unstable as the others. The mutants ranged from albino to very dark types, some being almost sterile, but others produced about as many conidia as the parent type. Thom (16) states: "The *P. notatum* series does mutate conspicuously". It was noted that the few mutants which appeared in soil and in soil infusion agar were darker than the parent, whereas most of those that arose on potato dextrose agar were much lighter in colour. The variants obtained in this experiment were essentially similar to those described in *P. notatum* by Raper and Alexander (13).

The fact that no mutants were isolated from the set of soil tubes that was not transferred suggests that transferring favoured the appearance of mutants.

Muskmelon *Fusarium:* This organism behaved much as in preceding experiments by Miller (9, 11). The wild type was not recovered from the potato dextrose agar slants and it was to a large extent displaced by mutants in soil infusion agar. In the soil tubes after 9 mo. it was still highly pure, only 1 tube in 8 yielding mutants. After 15 mo., however, all the transferred soil tubes yielded mutants, and in 2 instances the wild type had evidently been completely displaced since it was not re-isolated from the tubes. In the case of the soil tubes that were not transferred no mutants were isolated, even after 15 mo. This indicates that, as in *Penicillium*, transferring favours the appearance of mutations on this medium.

The mutants that were obtained from the transferred soil tubes (1 type per tube) showed a consistent difference from those that appeared on potato dextrose agar in that they were not greatly different morphologically from the wild type. As in the latter, aerial mycelium was abundant and no darkening of the submerged mycelium was evident. This is in contrast to the usual type of mutant that displaces the wild type on potato dextrose agar. Forms lacking aerial mycelium are commonly obtained on the latter medium, and some of them develop a pronounced dark pigmentation. A similar correlation of mutant types with culture medium has been noted above in *Septoria* and in *Penicillium*, but it was not as sharp as in the present case. This point will be considered further in the following section.

DISCUSSION

Raper and Alexander (13) describe some methods in general use for maintaining pure cultures of *Penicillium notatum*, including preservation of conidial suspensions in soil, and the lyophil process. The latter has been studied experimentally by the same authors (14), who concluded it to be applicable to a wide range of fungi. This method involves the suspension of conidia in sterile blood serum which is dispensed into small tubes, quickly frozen, vacuum-dried, and sealed under vacuum. Representative Hyphomycetes were found to be still viable after 20 mo. in lyophil tubes. When a large number of cultures are involved this method has many advantages over preservation in soil. However, the expensive equipment required would tend to make it impractical for branch laboratories where generally the number of cultures to be preserved is not high. The soil tube method as described here requires no special equipment and it is felt that the results presented will be of interest to workers who do not have access to lyophil equipment.

It is evident that a satisfactory technique for maintaining fungus cultures must satisfy 2 prerequisites: it must not only keep the organisms viable, but it must also be such that the original strains do not become displaced by mutants. Regarding the former, the relatively short period of viability of *Thielaviopsis* under these circumstances renders the technique of dubious value here, since transfers would have to be too frequent. *Septoria* was more promising, but would require at least two transfers per year. *Penicillium* and *Fusarium*, on the other hand, were found to endure a long period in dry soil. Miller (11) describes a case in which an isolate of *Fusarium* survived 3 yr. in dry soil and had suffered no loss in virulence at the end of this time. A further instance of longevity has been noted in the case of *Macrophomina Phaseoli* (Maubl.) Ashby. During a study on this organism by Hildebrand *et al.* (3) 2 soil tubes were inoculated with the Texas strain. Two years subsequently (in connection with the present study) platings were made and the organism was found to be still viable in both tubes.

With regard to the necessity of avoiding mutations, there can be no doubt that the soil tube technique is far superior to nutrient-rich agars. But it may be concluded that even on this medium transferring should be as infrequent as possible. Foster *et al.* (2) recommend holding to an absolute minimum the number of vegetative transfers of stock cultures to

prevent "biological degeneration" of *P. notatum*. Miller (9) found that frequent transferring accelerated the rate at which the wild type of the muskmelon *Fusarium* was displaced by mutants in potato dextrose agar.

The consistency with which the original types were displaced by mutants on agar media indicates that this effect is widespread, and possibly of fundamental biological significance. The process evidently does not happen in nature since the original isolates of these organisms are not found to include the "appressed" or "albino" or other strikingly different types. In this connection, a total of 64 single-chlamydospore isolates of *Thielaviopsis* was obtained from tobacco seedlings growing in flats of naturally infested soil. Diseased rootlets bearing abundant chlamydospores of the fungus as figured by Koch (6) were selected and crushed to form suspensions from which the single-spore isolates were obtained. All these appeared culturally identical. Miller (9, 11) describes similar instances in *Fusarium* in which the original isolates were identical or closely similar. That is, the organisms as isolated from nature display a much narrower range of variability than they do in artificial culture.

Perhaps the fact that these cultural variants were less virulent than the wild types accounts in part for the failure to isolate them from nature. But it must be noted that in a nutrient medium approximating that of the soil environment, i.e., tubes of sterilized soil, the organisms were genetically more stable than in nutrient-rich media. Further, the mutants that did appear in soil tubes and in soil infusion agar in most instances resembled the parent types more than did those that appeared in potato dextrose agar. It is possible, then, that the failure to isolate "off-types" from nature as often as from culture is in part the result of an effect of environment on the type of mutant that comes to predominate. This could be brought about in 2 ways: (1) environment may directly affect the type of mutations that arise; or (2) a selective effect may be involved whereby mutations occur at random, and those best adapted to a given medium rapidly achieve dominance. The second explanation would appear to account for certain cases of adaptation in bacteria. Luria and Delbrück (8) showed that mutations in cultures of *Escherichia coli* for resistance to bacteriophage were independent of the action of the phage, but where it was present only the progeny of the resistant cells survived. By an adaptation of the same technique, Demerec (1) showed that mutations of *Staphylococcus aureus* for resistance to penicillin occurred in the absence of the antibiotic. The indication is that these mutations were random and independent of any selective advantage. This may also be true of the fungi studied here, but it is not inconceivable that a given set of environmental conditions will favour the appearance of certain types of mutations.

The question is obviously of importance with regard to the problem of breeding for resistance, and stresses the value of research on the genetic behaviour of plant pathogens.

SUMMARY

The practice of maintaining fungus cultures in tubes of sterilized soil was compared with the usual laboratory technique of serial transfers on a nutrient-rich agar medium (potato dextrose agar) with regard to preventing loss of the original strains through mutations.

The organisms studied were: *Thielaviopsis basicola*, *Septoria glycines*, *Penicillium notatum*, and the muskmelon *Fusarium*.

Soil tube cultures of *Penicillium* and *Fusarium* were still viable after 15 mo. The limit of viability of *Septoria* in soil tubes was approximately 6 mo., but transferring to fresh tubes overcame this difficulty. *Thielaviopsis* did not survive more than 2 mo. in soil tubes.

It was consistently found that the original strains were soon displaced by mutant types on potato dextrose agar and in most instances on soil infusion agar. This happened rarely in soil cultures and could be avoided by not transferring.

When the soil culture method is to be employed, transfers should be no more frequent than is necessary to prevent loss of viability of the organism concerned.

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STUDIES ON RING-ROT OF POTATOES CAUSED BY *CORYNEBACTERIUM SEPEDONICUM*[1]

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It is well known that if viable cells of *Corynebacterium sepedonicum* (Spieck. and Kott.) Skaptason and Burkholder come in contact with the potato seed piece before it is planted, the ring-rot disease may develop. Efforts to control the disease are concerned mainly with preventing such contact. Occasionally cases of infection are observed which are not easily explained, and which lead one to suspect that infection took place while the plant was growing. Transmission of the pathogen to the vines by insects, and also its entrance by way of the roots, are two possibilities. With regard to the first, Kreutzer and McLean (2) found that although several genera of insects were able to accomplish inoculation of the vines, there would be scarcely any chance for the tubers to become infected during the crop season, because the spread of the bacteria in the plant is so slow. Obviously this factor would also practically eliminate the possibility of ring-rot bacteria from infected seed pieces reaching the leaves until too late in the season to be effectively transferred by insects. Entrance by way of the root system is the other possibility which was investigated by the author, and the results from this and other studies related to the spread of the ring-rot bacteria are presented in this paper.

Infection by Way of the Root System

The object of this experiment was to determine whether ring-rot bacteria are able to infect the potato plant by way of the roots.

Single eye sets from the variety Carter's Early Favourite were planted in moist sand. After 20 days, when the sets had sprouted and root growth had begun, the young plants were flooded out of the sand. Then the sets were removed from the base of the sprouts and the plants transplanted to crocks containing an aqueous nutrient solution. They remained here for 20 days, until the roots were 8 to 10 in. in length. At this stage, the roots were dipped momentarily in a suspension of *C. sepedonicum*, and then transplanted to the field. Four different treatments were employed, viz.: root tips dipped in the suspension of ring-rot bacteria; root tips abraded by rubbing with sand and then dipped in the bacterial suspension; the lower $\frac{2}{3}$ of the root system dipped in the suspension; and non-treated controls. Field soil was removed to a depth of about 10 in. so that the root system was accommodated without any contact of the inoculated and uninoculated portions. Moist granular soil was carefully sifted around the roots until the excavation was filled. There were 20 plants in each treatment. All tubers produced on these plants were harvested separately from each hill, and smears from each tuber were made and stained by the modified Gram stain method of Racicot, Savile, and Conners (3). These slides were examined under the oil immersion lens. The results are shown in Table 1.

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TABLE 1.—INFECTION OF POTATO PLANTS BY DIPPING PART OF THE ROOT SYSTEM IN AN AQUEOUS SUSPENSION OF *Corynebacterium sepedonicum*

Treatment	Diseased plants	Healthy plants	Dead plants[1]
	No.	No.	No.
Unwounded root tips dipped	11	8	1
Wounded root tips dipped	18	1	1
Lower ⅔ root system dipped	11	2	7
No treatment	0	20	0

[1] No tubers produced. Probably severe ring-rot.

According to the data in Table 1, the potato plants were readily infected by *C. sepedonicum* by way of the roots, and wounding the roots increased their susceptibility to infection.

INFECTION FROM OVER-WINTERED REMAINS OF RING-ROT POTATOES

If *C. sepedonicum* were able not only to survive in the remains of diseased tubers left in field soil, but also to enter the growing plant through its roots which penetrate or grow near these remains, this would constitute a danger readily appreciated. Several field experiments by others (1, 4) have not revealed any danger of infection from such over-wintered material, and only one (1) has indicated its possibility. Further tests under Alberta soil conditions were considered advisable.

Sets from certified stock of the variety Warba were planted in a block of 3 rows of 20 hills each. Those in the first row were planted at a depth of 4 in. directly above the remains of ring-rot potatoes placed 6 in. under the soil the previous fall, and in the second row they were planted in contact with them. The sets in the third row were planted in soil which had been soaked with a suspension of ring-rot bacteria the previous autumn. During late September several tubers from each hill were examined under the ultra-violet lamp, and stained smears were made from all suspected tubers and examined under the microscope. The results were negative in all cases.

THE SPREAD OF RING-ROT BACTERIA BY IRRIGATION WATER

Potato growers in the irrigation districts of Alberta frequently express the opinion that ring-rot bacteria are spread by irrigation water; consequently, there was need for experimental data on this important question.

Potato sets were planted in a block of 5 rows, each of 20 hills. The first row was planted with sets infected with ring-rot bacteria, and the other 4 rows with healthy sets from certified stock of the Warba variety. Early in August, when the plants were well advanced, a ditch 10 in. deep and 15 in. wide was opened between the diseased and healthy rows on one side of the block, and the ditch system continued between each of the remaining healthy rows. The roots of both diseased and healthy plants were severed and exposed along the borders of the ditch. This was immediately filled with water which flowed as in irrigation practice.

All tubers in the hills of the experiment, whether originating from healthy sets or diseased ones, were examined during late September. Smears were made from the tubers of all plants in the experiment and examined under the microscope. All of the plants from the infected sets were found to have developed the ring-rot disease. Not a single case of ring-rot infection was observed in the plants from healthy sets.

In the following year, another experiment, essentially like the one just described, was carried out with identical results.

Longevity of Ring-rot Bacteria on a Metal Surface

The cutting knife has been shown to be very important in spreading ring-rot bacteria, and the possibility that implements may carry infection has been recognized (1). The following tests were made to obtain additional information.

Late in September, 1943, the surface of 2 strips of iron, each comparable to the blade of a large knife, was thoroughly contamined by passing them through ring-rot tubers. They were then stored in an unheated field shed. During May, 1944, one of these contaminated knives was used to cut 20 sets from healthy tubers known to be free from ring-rot bacteria, and these were planted in the field. Similarly, in May, 1945, 25 sets were cut with the other contamined strip of metal. Each year, smears were made from all the tubers in each hill, and examined under the microscope. Only 2 of the 20 plants in the 1944 tests were found to be positive for ring-rot bacteria, and of the 25 plants in 1945 all were negative.

Dry Heat for Decontaminating Potato Sacks Infested with Ring-rot Bacteria

A laboratory experiment was undertaken to determine the capacity of the ring-rot bacteria on used potato sacks to survive dry heat. Preliminary work showed that without special equipment for rapid evaporation it required at least 5 hr. at 50° C. for wet jute sacks to dry. The use of a wet disinfectant during winter, when the used sacks accumulate, would not be convenient.

For this experiment a jute sack was cut into pieces 1 ft. sq., and these were placed in 1 l. Erlenmeyer flasks, and steam sterilized for 1 hr. at 15 lb. pressure. One ml. of an aqueous suspension of *C. sepedonicum* from pure culture was applied by pipette to the sacking in each of the flasks. The flasks (in fours) were then put into constant temperature ovens at various temperatures for different periods of time, viz.: 47° C. for 3 and 16 hr.; 50° C. for 4 hr.; 58° C. for 2 and 3 hr.; and 68° C. for 2 and 3 hr. Four flasks were untreated as controls. After treatment, 100 ml. of sterilized water was added to each flask and the sacking soaked in this for 2 hr. A sample of the liquid was withdrawn aseptically and one ml. portions added to tubes of liquid medium (4), recommended for the cultivation of this pathogen. After incubation for one week at 20° to 25° C., smears were made from the liquid in the tubes, stained, and examined under the microscope. There was bacterial growth in the control tubes, but not in the other series. Apparently all treatments were sufficient to destroy the ring-rot bacteria on the sacking.

The results of the foregoing experiment were checked by another experiment. Marked areas on pieces of jute sacking were soaked in a suspension of *C. sepedonicum* made from macerated ring-rot tubers. The edges of these pieces of sacking were attached to a line suspended within a small chamber of approximately 70 cu. ft., equipped with electric heating units thermostatically controlled. At the end of 3.5, 4, 4.5 and 5 hr., respectively, at 50° C., pieces of the sacking were removed from the chamber and the marked area moistened with sterile water. Then, in each instance, 12 freshly cut sets of Carter's Early Favourite potatoes were rubbed over the area and immediately planted in the greenhouse in pots of moist soil. The controls consisted of 12 healthy, untreated sets, and 12 sets rubbed on sacking soaked in a suspension of the ring-rot bacteria before it was exposed to the heat treatment.

After 46 days, when the plants were about 9 in. high, 6 of them in each lot were examined for the presence of ring-rot bacteria. Smears were made on microscope slides from the stems as they emerged from the sets, from the stems at 1.5 in. above the sets, from the roots arising at the base of the stems, from the new tubers, and from the sets themselves. The preparations were stained and examined under the oil immersion lens.

C. sepedonicum was observed on all slides prepared from the inoculated controls. It was very numerous in the sets, and had migrated to the stems. The new tubers, which were then just beginning to form, were apparently free, as were also the stems at 1.5 in. above the sets. The pathogen was not found in stems roots, tubers, or sets in any of the other treatments.

After a further 34 days, smears were made from the remaining 6 plants in each series. At this time, the pathogen was abundant in the inoculated controls, where it had spread in the stems at least as far as the second node, and into the roots growing at the base of the stems. It also had reached the new tubers of 2 of the 6 plants.

All the other treatments were negative, with the possible exception of 1 plant in the 5.0-hour series, in which small, Gram positive bacteria were found at the base of the stem. It is almost certain, however, that these bacteria were invaders from the soil.

DISCUSSION

The results cited are evidence that ring-rot bacteria can enter the potato plant through its roots. However, it must be remembered that the infection which was secured experimentally developed under conditions where the bacteria in contact with the roots were very abundant. Although no infection was observed in the irrigation field experiments described, one would not on the basis of this evidence claim that living bacteria could not be carried in irrigation water and infect the roots of potato plants. The data merely indicate that this would be a remote possibility.

With regard to persistence of the pathogen in the soil during the winter, the evidence secured in this study confirms the negative data obtained by other workers (1). Therefore, it is safe to conclude that the bacteria are not likely to survive the winter in the soil or in the remains of diseased potatoes left in the field, since in this experiment ample oppor-

tunity for infection was afforded and none occurred. On the other hand, it is interesting to note that the bacteria remained viable from September to May on the dry surface of metal stored in an unheated field shed.

These studies have also shown that the ring-rot bacteria on used potato sacks can be killed by dry heat maintained at 50° C. for at least 4 hr., and it would seem that this method could be adapted to practical use. However, in practice it would be wise to increase both the temperature and time factors, if possible. In any case, it is essential that the sacks be hung from their edges in the chamber in such a manner that the heat will penetrate quickly and freely to all parts.

SUMMARY

When the injured and uninjured root tips of 40-day-old potato plants were momentarily dipped in a suspension of *Corynebacterium sepedonicum*, and then transplanted to the field, ring rot developed in the tubers of these plants.

No ring rot developed in plants from healthy sets placed in contact with, or adjacent to, overwintered diseased tubers, or when healthy sets were planted in field soil heavily infested the previous fall with an aqueous suspension of ring-rot bacteria.

Ring-rot bacteria on jute sacking, suspended for 3.5 to 5.0 hr. in a chamber heated at 50° C., were killed.

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THE RELATIVE VALUE OF CERTAIN GRASS-LEGUME MIXTURES FOR HAY AND PASTURE IN SHORT-TERM ROTATIONS[1]

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Grass-legume mixtures in short-term rotations in eastern Canada are usually treated in one of the following ways:— A—Hay crops are taken during the first and second years following seeding and pastured thereafter; B—A hay crop is taken the first year only following seeding and pastured thereafter; C—Pastured continuously beginning the year following seeding. Also in many districts the aftermath provides late summer and early fall grazing. With a view to determining the effect of these various practices on the total yield of dry matter per acre, the percentages and total yield of protein per acre, and upon the relative persistence of the various species, three groups of plots were laid down in 1939.

MATERIALS AND METHODS

Each group of plots contained the same mixtures, nine in all. The plots in group A were harvested for hay in 1940 and 1941 and were clipped 5 times during 1942 to simulate grazing. Those in group B were harvested for hay in 1940 only, and clipped 5 times annually to simulate pasture during the following 2 years. The plots in group C were treated as pasture by clipping 5 times annually throughout the three years of this experiment.

The mixtures were sown in all cases with a nurse crop of oats which was removed as hay. Each group contained 3 replicated plots of each of the 9 mixtures. The plots were $41' \times 6'$ in size and were arranged in randomized blocks.

The harvesting of the plots was so arranged that those treated as hay were cut twice, and those treated as pasture were cut 5 times during each season to simulate grazing. All the plots in any one group were harvested on the same day with a small tractor mower equipped with a 42″ cutting bar. Borders were removed before the crop was harvested. The final area harvested was 1/363 acre. Green yields were recorded and representative samples were taken for dry matter determination. These were later used for protein analyses.

Just prior to each cutting, all plots were examined carefully and estimates were made of the percentage contributed by each species to the total yield. Occasional checks on the estimates were made by taking small samples of the harvested material from which the individual species were separated, dried and weighed. It was found that with some practice the estimation of percentage contribution made by the different species could be done fairly accurately. In no case was the error of estimation as much as 5%.

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The following mixtures were included in each group:

TABLE 1—RATES OF SEEDING MIXTURES OF GRASSES AND LEGUMES

Mix No.	Species in lb. per acre
1	Timothy 8, alfalfa 4, red clover 4, alsike 2, Kentucky blue 1½, Canada blue 1½, red top 2, New Zealand wild white clover 1.
2	Brome 16, alfalfa 4, red clover 4, alsike 2, Kentucky blue 1½, Canada blue 1½, red top 2, New Zealand wild white clover 1.
3	Slender wheat grass 12, alfalfa 4, red clover 4, alsike 2, Kentucky blue 1½, Canada blue 1½, red top 2, New Zealand Wild White Clover 1.
4	Timothy 8, alfalfa 4, red clover 4, alsike 2.
5	Brome 16, alfalfa 4, red clover 4, alsike 2.
6	Slender wheat grass 12, alfalfa 4, red clover 4, alsike 2.
7	Timothy 10, alfalfa 8.
8	Brome 16, alfalfa 8.
9	Slender wheat grass 12, alfalfa 8.

NOTE: Commercial seed was used in all mixtures. Alfalfa variety was "Grimm" and red clover was "Ottawa" double cut variety.

RESULTS

YIELD OF DRY MATTER

The yields of dry matter produced by the different mixtures in the 3 groups are shown in Table 2, and the analysis of variance in Table 3.

These data show that the average yield of dry matter of all mixtures in group A in 1940 was 523 pounds per acre higher than in group B which was also harvested for hay that year. This difference, however, was not statistically significant and requires no further comment. Group C, which was treated as pasture continuously throughout the test, in 1940 produced only 61.33% as much as group A and 66.63% as much as group B, both of which were harvested for hay that year.

During 1941, the mixtures in group A were again cut for hay whereas those in group B were treated as first year pasture following the hay crop which had been harvested in 1940, while group C was treated as pasture for the second year. In this case, the mixtures in group B produced an average of 71.35% and those in group C only 55.22% of those in group A.

In 1942 all 3 groups were treated as pasture. In group A the pasture followed 2 years of hay. In group B it followed 1 year of hay and 1 year of pasture, while in group C it followed 2 years of pasture. There was no difference in the amount of herbage produced by the mixtures in groups B and C which yielded only 47.58 and 48.57% respectively of group A.

Referring now to the annual yields of group A we observe that the yield of pasture in 1942 was higher than the yield of hay secured from these same plots in 1941. On the other hand, the yield of pasture in group B during 1942 was only 70.37% of the yield of pasture secured in 1941 from the same plots. The first year pasture yields from group C were just equal

TABLE 2.—YIELDS OF DRY MATTER IN POUNDS PER ACRE OF NINE MIXTURES TREATED IN THREE DIFFERENT WAYS

GROUP A = hay, hay, pasture; GROUP B = hay, pasture, pasture; GROUP C = pasture, pasture, pasture

Mixture No.	Species in pounds per acre	Group A			Group B			Group C			Ave. by groups			Ave. by mixtures
		1940	1941	1942	1940	1941	1942	1940	1941	1942	A	B	C	
1	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1	6742	4577	5654	5960	3841	2729	4539	2691	2495	5658	4177	3242	4359
2	Brome 16, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1	7192	5040	6390	6966	3621	2456	4324	2831	2536	6207	4348	3230	4595
3	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1	6867	5871	5193	5522	3780	3079	4050	2291	2650	5977	4127	2997	4367
4	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2	6628	5275	5541	6521	3413	2963	4105	3441	3252	5815	4299	3599	4570
5	Brome 16, alfalfa 4, red clover (early) 4, alsike 2	6606	5662	4525	5647	3355	2677	4576	2387	2595	5598	3893	3186	4226
6	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2	6594	5444	6025	6176	3518	2544	4241	2440	3009	6021	4079	3230	4443
7	Timothy 10, alfalfa 8	5789	6288	6335	5889	5009	3110	3554	4392	3556	6137	4669	3834	4880
8	Brome 16, alfalfa 8	6090	6458	6968	6026	4860	3206	3210	3928	2751	6505	4697	3296	4833
9	Slender wheat grass 12, alfalfa 8	6676	6375	7166	5772	4994	2844	3697	3755	3288	6739	4537	3580	4952
	Average yield of dry matter	6576	5666	5977	6053	4043	2845	4033	3129	2904	6753	4314	3355	

Minimum difference required for significance at the 5% point: Between mixtures in all groups 424 lbs.; between mixtures in any one group 735 lbs.; between groups (3-year averages) 245 lbs.; between years (average of all groups) 245 lbs.; between years (in any one group) 425 lbs. per acre.

TABLE 3—ANALYSIS OF VARIANCE FOR ALL YIELD DATA (1941 TO 1942 INCLUSIVE)

Source of Variance	Degrees of freedom	Mean Square	F Value obtained	F value for 5%	F value for 1%
Mixtures	8	2,508,175	3.96	1.98	2.60
Replicates	6	2,717,585	4.29	3.04	4.71
Groups	2	145,133,032	229.37	3.04	4.71
Years	2	50,010,585	80.62	3.04	4.71
Mixtures × Groups	16	1,157,261	1.83	1.69	2.09
Mixtures × Years	16	3,371,963	5.33	1.69	2.09
Groups × Years	16	3,039,362	4.80	1.69	2.09
Error	180	632,747			

to the first year pasture yields from group B. The pasture yields from the mixtures in group A in 1942 were significantly higher than the pasture yields secured from the groups B and C for any year during the test. However, it will be noted that there was a decided decrease in yield of herbage during the second pasture year in groups B and C. In the third pasture year of group C yields were maintained at approximately the same level as for the second pasture year. Thus the plots in Group A (2 years of hay and 1 of pasture) produced a total of 20,259 pounds of dry matter per acre during the 3-year period. Plots in group B (1 year of hay and 2 years of pasture) produced only 12,942 pounds while plots in group C (3 years of continuous pasture) produced only 10,065 pounds of dry matter per acre during the three-year period. On relative basis the 3-year total yields were 100.00, 63.88 and 49.68 for groups A, B and C, respectively.

Insofar as the yields of different mixtures are concerned, and on the basis of least difference for significance of 735 pounds between mixtures in any one group, the following conclusions may be drawn. In group A (2 years of hay and 1 of pasture) mixture 9 was significantly higher in yield than mixtures 3, 4, 1 and 5, while mixture 8 was significant over mixtures 1 and 5. In group B (1 year of hay and 2 years of pasture) there were no significant differences between the yields of mixtures. In group C (3 years of pasture only) mixture 7 was significantly higher in yield than mixture 3. However, if we examine the last column in Table 2 we see that mixtures 7, 8 and 9 were significantly higher in yield than mixtures 1, 3, 5, and 6. Hence, under the conditions of this experiment simple mixtures of alfalfa and one tall growing grass (timothy, brome or slender wheat-grass) produced more than the more complex mixtures containing the "bottom" species. This may be summarized as follows:

Type of mixture	3-year average all groups lb. dry matter per acre
Legumes, tall grasses and "bottom" species	4440
Legumes and tall grasses	4413
Alfalfa only and tall grasses	4888

TABLE 4.—YIELDS OF PROTEIN IN POUNDS PER ACRE OF NINE MIXTURES TREATED IN THREE DIFFERENT WAYS

GROUP A = hay, hay, pasture; GROUP B = hay, pasture, pasture; GROUP C = pasture, pasture, pasture

Mixture No.	Species in pounds per acre	Group A			Group B			Group C			Ave. by groups			Ave. by mixtures
		1940	1941	1942	1940	1941	1942	1940	1941	1942	A	B	C	
1	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	1026	677	1221	908	734	442	993	412	383	975	695	596	755
2	Brome 16, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	1088	690	1440	912	671	395	885	488	404	1073	659	592	775
3	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	978	784	1112	879	802	529	852	373	426	958	737	550	748
4	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2.	982	676	1236	999	732	550	891	681	544	965	760	705	810
5	Brome 16, alfalfa 4, red clover (early) 4, alsike 2.	909	742	970	818	684	484	1032	449	487	874	662	656	731
6	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2.	973	782	1445	897	754	494	943	451	520	1067	715	638	807
7	Timothy 10, alfalfa 8	893	934	1423	897	1166	552	801	943	628	1083	872	791	915
8	Brome 16, alfalfa 8	836	898	1659	827	1179	621	744	806	499	1131	876	683	897
9	Slender wheat grass 12, alfalfa 8	1017	932	2050	839	1188	532	842	796	597	1333	853	745	977
	Average yield of protein	967	791	1395	886	879	511	887	600	499	1051	759	662	

TABLE 5.—WEIGHTED PERCENTAGE OF PROTEIN IN NINE DIFFERENT MIXTURES TREATED IN THREE DIFFERENT WAYS

GROUP A = hay, hay, pasture; GROUP B = hay, pasture, pasture; GROUP C = pasture, pasture, pasture.

Mixture No.	Species in pounds per acre	Group A			Group B			Group C			Seasonal average All groups
		1940	1941	1942	1940	1941	1942	1940	1941	1942	
1	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	15.32	14.79	21.60	15.23	19.11	16.20	21.88	15.31	15.35	17.19
2	Brome 16, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	15.13	13.69	22.54	13.09	18.53	16.08	20.47	17.24	15.93	16.97
3	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	14.24	13.35	21.41	15.92	21.22	17.11	21.04	16.28	16.08	17.41
4	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2	14.82	12.82	22.31	15.32	21.45	18.56	21.71	19.79	16.73	18.17
5	Brome 16, alfalfa 4, red clover (early) 4, alsike 2	13.76	13.10	21.44	14.49	20.39	18.08	22.55	18.81	18.77	17.51
6	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2.	14.76	14.36	23.98	14.52	21.43	19.42	22.24	18.48	17.28	18.50
7	Timothy 10, alfalfa 8	15.43	14.85	22.46	15.27	23.28	17.75	22.54	21.47	17.66	18.83
8	Brome 16, alfalfa 8	13.73	13.91	23.81	13.73	24.26	19.37	23.18	20.52	18.14	18.66
9	Slender wheat grass 12, alfalfa 8	15.23	14.62	28.61	14.54	23.79	18.71	22.78	21.20	18.16	19.87
	Average	14.70	14.02	23.13	14.69	21.50	17.93	22.04	19.18	17.19	

Percentage and Yield of Protein

All samples collected for the determination of yields and percentages of dry matter were analysed for crude protein content. The yield of dry matter secured from each cutting was multiplied by the percentage of crude protein in the sample in order to find the yield of protein per acre from different mixtures in different groups. Seasonal yields of protein were thus calculated as shown in Table 4.

It may be seen that the average yield of protein per season was the highest in group A, while it was the lowest in group C. When we examine the annual yield of protein within each group we note that pasture in group A (1942) contained 1,395 pounds of protein while group B gave 879 pounds in the first year pasture (after 1 year of hay) and only 511 pounds in the second year of pasture.

The average yield of protein from different mixtures was higher in the more simple grass-alfalfa mixtures like Nos. 7, 8 and 9. This may be explained on the basis of the relatively high percentage of legumes contributing to the yield of these mixtures. The yields of protein from different types of mixtures are given below.

Type of mixture	3-year average all groups lb. protein per acre
Legumes, tall grasses and "bottom" species	759
Legumes and tall grasses	783
Alfalfa only and tall grasses	930

By using the dry matter yield data in Table 2, and the yield of protein data in Table 4, it was possible to calculate the average weighted percentage of protein for each year for the mixtures in the 3 groups. These data are shown in Table 5. It was found that the average percentage of protein in hay in group A was 14.70 in 1940 and 14.02 in 1941. Pasture clippings in the following year contained an average of 23.13% protein. Hay in group B in 1940 contained 14.69% protein followed by 21.50 and 17.93% protein in pasture clippings in 1941 and 1942 respectively. The percentage of protein in the pasture clippings from group C was 22.04, 19.18 and 17.19 for 1940, 1941 and 1942 respectively. It may be seen also that pasture clippings from group A (previously in hay for 2 years) were very much higher in percentage protein than pasture clippings from the other groups in the same year. This significant increase in the protein content in the pasture of group A may be explained partly by the fact that it contained considerably more alfalfa than the other groups.

The simple alfalfa and tall-grass mixtures contained a higher percentage of protein than the other type of mixtures in this test. Under the 3 different treatments over the 3 years the percentages of protein were as follows:

Type of mixture	3-year average all groups weighted percentages protein
Legumes, tall grasses and "bottom" species	17.19
Legumes and tall grasses	18.06
Alfalfa only and tall grasses	19.12

TABLE 6.—PERCENTAGE CONTRIBUTION OF ALFALFA IN NINE MIXTURES TREATED IN THERE DIFFERENT WAYS

GROUP A=Hay, hay, pasture; GROUP B=hay, pasture, pasture; GROUP C=pasture, pasture, pasture

Mixture No.	Species in pounds per acre	Group A			Group B			Group C		
		1940	1941	1942	1940	1941	1942	1940	1941	1942
1	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	17	39	47	20	32	5	22	18	7
2	Brome 16, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1	12	23	54	22	28	5	19	21	6
3	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	17	37	32	19	27	7	19	16	13
4	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2.	23	40	59	18	33	25	30	37	21
5	Brome 16, alfalfa 4, red clover (early) 4, alsike 2.	17	22	32	20	34	12	24	23	11
6	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2.	21	53	66	19	59	15	25	32	14
7	Timothy 10, alfalfa 8	74	67	53	67	79	17	46	64	24
8	Brome 16, alfalfa 8	75	81	67	65	76	14	62	59	14
9	Slender wheat grass 12, alfalfa 8.	76	86	90	66	80	17	45	46	17
	Average	37	51	56	35	50	13	32	35	14

Botanical Analysis

The percentage contributed by different species to the total yield was estimated in every plot just before it was cut. These data are summarized in Tables 6 to 8.

Alfalfa was seeded in every mixture and for this reason is one of the most important species under study in this experiment. It is shown in Table 6, that in general there is a gradual increase in the percentage contribution of alfalfa in all mixtures in group A from 1940 to 1942. This was particularly so in the more complex mixtures containing other legumes and bottom grasses. The average percentage of alfalfa in the 9 mixtures in group A was 37, 51 and 56 in 1940, 1941 and 1942, respectively. The proportion of alfalfa in group B was the same as in Group A during the

Table 7.—Percentage contribution of legumes other than alfalfa in nine mixtures treated in three different ways

Group A = hay, hay, pasture; Group B = hay, pasture, pasture; Group C = pasture, pasture, pasture

Mixture No.	Species in pounds per acre	Group A			Group B			Group C		
		1940	1941	1942	1940	1941	1942	1940	1941	1942
1	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	70	22	3	61	18	5	38	11	0
2	Brome 16, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	69	20	1	54	8	14	41	9	0
3	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	56	18	0	62	11	0	61	10	0
4	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2.	70	17	0	71	28	0	53	17	6
5	Brome 16, alfalfa 4, red clover (early) 4, alsike 2.	63	21	0	65	23	0	51	21	0
6	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2.	67	23	0	64	20	0	45	11	0
7*	Timothy 10, alfalfa 8	0	0	0	0	0	0	0	0	0
8*	Brome 16, alfalfa 8	0	0	0	0	0	0	0	0	0
9*	Slender wheat grass 12, Alfalfa 8	0	0	0	0	0	0	0	0	0
	Average	44	13	0	42	12	2	32	9	1

* Alfalfa was the only legume seeded.

first 2 years but dropped appreciably in the third year, following 1 year of simulated pasture, being 35, 50 and 13% in 1940, 1941 and 1942 respectively. The percentage contributed by alfalfa in group C, however, was slightly lower in the first year of the test than in the other 2 groups. It remained approximately the same during the second year, but showed a marked decrease in the third year of pasture. The percentages of alfalfa were 32, 35 and 14 in 1940, 1941 and 1942, respectively.

Comparing groups A, B and C, it is quite evident that the proportion of harvested forage contributed by alfalfa was maintained for the 3-year period in group A, but showed a decided decrease in groups B and C during the years the plots were clipped 5 times annually to simulate pasture.

The percentages of legumes other than alfalfa are given in Table 7. It is clearly seen that there was a good proportion of legumes in each group

TABLE 8—PERCENTAGE CONTRIBUTION OF GRASSES SEEDED IN NINE MIXTURES TREATED IN THREE DIFFERENT WAYS

GROUP A=hay, hay, pasture; GROUP B=hay, pasture, pasture; GROUP C=pasture, pasture, pasture

Mixture No.	Species in pounds per acre	Group A			Group B			Group C		
		1940	1941	1942	1940	1941	1942	1940	1941	1942
1	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	13	39	50	19	50	90	40	71	93
2	Brome 16, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	19	47	45	26	64	81	40	70	94
3	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2, Kentucky Blue 1½, Canada Blue 1½, red top 2, New Zealand wild white clover 1.	27	35	68	19	62	93	20	74	87
4	Timothy 8, alfalfa 4, red clover (early) 4, alsike 2.	7	43	41	11	39	75	17	41	73
5	Brome 16, alfalfa 4, red clover (early) 4, alsike 2.	20	57	68	15	43	88	25	56	89
6	Slender wheat grass 12, alfalfa 4, red clover (early) 4, alsike 2.	12	24	34	27	21	95	30	57	86
7	Timothy 10, alfalfa 8	26	33	47	33	21	83	54	36	76
8	Brome 16, alfalfa 8	25	19	33	35	24	86	38	41	86
9	Slender wheat grass 12, alfalfa 8.	24	14	10	34	20	83	55	54	83
	Average	19	35	44	24	38	85	35	56	85

in 1940. This was reduced to less than $\frac{1}{3}$ by 1941. By 1942, these legumes did not contribute to the total yield. The largest contribution during 1940 was made by red clover and to some extent by alsike in those plots where these species were seeded (see Table 1). Red clover being a biennial, however, almost completely disappeared from all the plots in 1941. The percentages of legumes in 1941, shown in Table 7, are for alsike and white clover. Even these legumes failed to contribute appreciably to the yields during 1942, irrespective of the treatment. However, white clover was present to some extent in the bottom in those mixtures where it was seeded, but was too short to be mowed and picked up at the time of harvest. It is true that white clover would have increased the palatability and to some extent the percentage of protein in the herbage, had it been grazed, but it is doubtful if this contribution would have been significant.

The percentages of grasses contributing to the yield are given in Table 8. It is readily seen that the contribution of the grasses increased from 1940 to 1942, but that the highest increase took place in 1942 in group B and group C. This, as should be expected, was in the reverse proportion to the legumes present during that period. The average percentage contributed by the grasses was 18, 35 and 44 in groups A: 24, 38 and 85 in groups B, and 35, 56 and 85 in groups C during 1940, 1941 and 1942 respectively. Thus the average percentage of grass in the pasture plots in 1942 was 44 after 2 years of hay (group A), 85% after 1 year of hay and 1 of pasture (group B), and 85% after 2 years of pasture (group C). The longer the mixtures were treated as hay, the longer the alfalfa persisted and thus there was a smaller percentage contribution made by the grasses in those plots.

SUMMARY

The results show that under the conditions of this experiment the mixtures used in this test produced over a period of 3 years a total of 20,259 pounds of dry matter and 3,053 pounds of protein per acre when used as hay for 2 years (2 cuts annually) and as pasture for 1 year (5 clippings annually to simulate grazing). The same mixtures produced a total of 12,942 pounds of dry matter and 2,277 pounds of protein per acre when used as hay for 1 year and as pasture for 2 years. However, when used continuously as pasture for 3 years these mixtures produced a total of only 10,065 pounds of dry matter and 1,986 pounds of protein per acre.

The amount of herbage produced in the third year of the experiment after 2 years of hay was significantly higher than after 1 year of hay and 1 year of pasture or after 2 years of pasture.

In a system of short-term rotation in this test the so-called hay species, including tall-growing grasses and alfalfa, produced a greater yield of dry matter and a greater amount of protein per acre than mixtures containing the so-called "bottom" species.

Botanical analyses of all plots show that alfalfa was being maintained better and contributed more to the total production when used for hay for 1 or 2 years prior to clipping to simulate grazing than under continuous clipping to simulate grazing beginning the year following seeding. Conversely, the grasses contributed the greatest portion of the yield under continuous clipping treatment to simulate pasture conditions.